In vitro translocation of bacterial proteins across the plasma membrane of *Escherichia coli*

(protein export/cell-free transcription-translation/inverted vesicles/posttranslational translocation)

MATTHIAS MÜLLER* AND GÜNTER BLOBEL

Laboratory of Cell Biology, The Rockefeller University, New York, NY 10021

ABSTRACT Precursors to two periplasmic proteins and one outer membrane protein were synthesized in a membrane-free extract from *Escherichia coli* programmed with plasmid DNA. In the presence of inverted plasma membrane vesicles from *E. coli* up to 25% of the precursor molecules were converted into their mature forms. Using externally added proteinase K as a probe, we found the processed proteins segregated within the membrane vesicles. By the same criteria, a small amount of each precursor also proved to be translocated, indicating that translocation and signal sequence cleavage are not necessarily coupled processes. Furthermore, we present conclusive evidence that the translocation step can occur posttranslationally even as late as 60 min after the beginning of translation.

Translocation of secretory proteins across the eukaryotic rough endoplasmic reticulum (RER) and the prokaryotic plasma membrane share common features. The information for targeting secretory and certain integral membrane proteins to these two membranes resides in a discrete segment of the polypeptide, termed the signal sequence (also known as leader or pre sequence). For most (but not all) examples of secretory proteins so far studied the signal sequence is located at the NH$_2$ terminus and is cleaved during or after translocation. The informational content of the signal sequence of *prokaryotic* and *eukaryotic* secretory proteins appears to be similar as either group of proteins can be translocated across the other, heterologous membrane (1–6). These findings suggested that the translocation machinery of these two membranes might be similar as well.

In the case of the RER, some components of the translocation machinery have been isolated. This was possible because *in vitro* translocation systems had been developed that were mRNA- and membrane-dependent and that reproduced chain translocation with fidelity (7, 8). Fractionation of this system led to the isolation and characterization of two components: an 11S ribonucleoprotein (9, 10), termed the signal recognition particle (SRP), and a 72-kDa integral membrane protein of the RER, termed the SRP receptor (11) or the docking protein (12). It was demonstrated that the signal sequence is recognized by SRP as soon as it emerges on the surface of the ribosome (13, 14). This recognition is accompanied by a dramatic increase in the affinity of SRP for the ribosome and a site-specific elongation arrest (13, 14). The SRP receptor displaces SRP from the ribosome and thereby releases the elongation arrest (15). In doing so, the SRP receptor, however, does not itself bind to the ribosome (15). Subsequent events, in particular, those involved in the passage of the chain across the microsomal membrane, remain to be deciphered.

Translocation across (or integration into) the prokaryotic plasma membrane has also been reproduced in cell-free sys-

tems (16–22). However, these systems are less developed and characterized than the eukaryotic one described above. Genetic studies in *Escherichia coli* have led to the isolation of secretion mutants (reviewed in refs. 23 and 24) with a pleiotropic export-defective phenotype, clearly indicating the existence of bacterial gene products necessary for translocation. The precise function of these products, however, has so far not been elucidated. As a first step toward a biochemical identification of these genetically defined components, we describe here a simple and dependable *in vitro* system. This translocation system consists of a high-speed supernatant prepared from an *E. coli* S-30 and is dependent on the addition of DNA or mRNA and translocation-competent inverted vesicles derived from the *E. coli* plasma membrane. Two periplasmic proteins and one outer membrane protein have been shown to be translocated with fidelity. Moreover, it was demonstrated that passage of these proteins through the membrane can occur posttranslationally.

METHODS

Cell Extract. An S-30 was prepared according to Zubay (25) from the *E. coli* strains MRE600 (26) and MZ9 (Δ *lac* Δ *phoA phoB⁺ phoR⁻*). MZ9 was grown in a medium containing (per liter) 5.6 g of NaCl, 1 ml of 1 M NaOH, 0.8 g of yeast extract, 9 g of bactotryptone, and 0.8 g of glucose. For breakage of the cells a 1:1 (wt/vol) suspension was passed twice at 4000 psi (1 psi = 6.89 kPa) through a French pressure cell (MRE600) or treated with a Branson sonifier (MZ9). Inclusion of 0.5 mM phenylmethylsulfonyl fluoride (PhCH$_2$SO$_2$F) into the homogenization buffer drastically reduced nonspecific proteolysis otherwise occurring during translation at 37°C, as judged by a loss of acid-insoluble radioactive material. A membrane-free S-135 was obtained by centrifuging 175 μl of S-30 per tube in the A-100/18 rotor of a Beckman Airfuge for 13.2 min at 4–6°C and at 30 psi (135,000 × g) and collecting the top 120–135 μl.

Inverted Plasma Membrane Vesicles (INV). INV were prepared from MRE600 cells grown as described (25) with the addition of 1% bactotryptone. All subsequent steps were carried out at 2–4°C. Cells were resuspended (1 g/ml) in buffer A (50 mM triethanolamine acetate, pH 7.5/250 mM sucrose/1 mM EDTA/1 mM dithiothreitol/0.5 mM PhCH$_2$-SO$_2$F) and passed through the French press as described above. The homogenate was freed of cell debris (5 min at 3000 × g) and the resulting supernatant was centrifuged for 2 hr at 150,000 × g$_{av}$. The pellet was resuspended in buffer B (buffer A without EDTA and PhCH$_2$SO$_2$F) by using a loosely fitting Dounce homogenizer. Aggregated material was removed at 12,000 × g and the clear supernatant (20–30 A$_{280}$ units/ml, determined in 2% NaDodSO$_4$) representing

Abbreviations: SRP, signal recognition particle; PhCH$_2$SO$_2$F, phenylmethylsulfonyl fluoride; INV, inverted plasma membrane vesicle(s); RER, rough endoplasmic reticulum.
*Present address: Biochemisches Institut der Universität Freiburg, Hermann-Herder-Str. 7, D-7800 Freiburg, F.R.G.

"crude" INV was subaliquoted, frozen in liquid N_2, and stored at $-80°C$. If outer membrane-free INV were to be prepared, the $150,000 \times g_{av}$ pellet was resuspended in buffer A and layered on top of a sucrose-step gradient in buffer A according to ref. 27. After centrifugation, INV were collected, pelleted at $150,000 \times g_{av}$, and resuspended and stored as described above ($10-15$ A_{280} units/ml).

In Vitro **Translation.** A typical $25-\mu l$ translation contained 40 mM triethanolamine acetate (pH 7.5), 140 mM KOAc, 11 mM $Mg(OAc)_2$, 20 mM NH_4OAc, 0.1 mM EDTA, 0.8 mM spermidine, 3.2% (wt/vol) polyethylene glycol 6000–8000, 2.5 mM ATP, 0.5 mM GTP, 10 mM KOH to neutralize ATP and GTP, 2 mM dithiothreitol, 12 mM phospho*enol*pyruvate, 8 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 40 μM each of 19 amino acids, $10-15$ μCi of L-[^{35}S]methionine (1 Ci = 37 GBq), $3-4$ μl of S-135, and 4 μl of transcription mix (4) containing plasmid DNA, RNA polymerase, and transcribed RNA. Protein synthesis was due both to translation of the transcribed RNA and to transcription-translation of the DNA template as the efficiency of synthesis was found to be reduced by addition of the RNA polymerase inhibitor rifampicin to the translation mix (data not shown). Incubation was at 37°C. The optimal time (shortly before the plateau of [^{35}S]methionine incorporation was reached) was determined for each new batch of S-135 (usually 30–45 min). The reaction was stopped with 5% trichloroacetic acid or by adding proteinase K (see figure legends). The translation products of a $25-\mu l$ reaction were separated by electrophoresis in 10–15% polyacrylamide gradient gels in NaDodSO$_4$ and could easily be visualized by an overnight fluorography.

Plasmid DNA was isolated (4) from plasmid pHI-1 (a generous gift of H. Inouye and J. Beckwith) or from plasmid-pLB 7012B (a generous gift of S. Benson). Plasmid pHI-1 contains the gene for alkaline phosphatase (28) and codes in addition for truncated β-lactamase (4). Plasmid pLB 7012B contains the *lamB* gene under synthetic promoter control (29).

RESULTS

The cell-free *E. coli* translation system used for the experiments described in this paper contained an S-30 that had been preincubated (25) in order to eliminate endogenous mRNAs, presumably by readout and degradation. Remaining polysomes were removed by a high-speed centrifugation ($135,000 \times g$) resulting in an S-135 that, in addition, and unlike an S-30, was completely free of translocation-competent INV. Therefore no "backround" translocation occurred in the translation system containing an S-135 rather than an S-30. The relatively modest extent of translocation obtained by the addition of INV (see below) therefore yields a better signal-to-noise ratio.

Protein synthesis was initiated by transcripts (see *Methods*) from two different plasmids. As shown in lane 1 of Fig. 1A, plasmid pHI-1 directed synthesis primarily of the precursors of two periplasmic proteins, alkaline phosphatase (closed arrow) and a truncated β-lactamase (closed arrowhead). Pre-alkaline phosphatase was identified by the dependence of its expression on a repressor-negative background in the S-135 (strain MZ9) (data not shown). The synthesis of the precursor of the truncated β-lactamase had first been demonstrated when pHI-1 transcripts were translated in a cell-free system made from wheat germ (4). Plasmid pLB 7012B directed synthesis of, among other proteins, the precursor of LamB, an integral membrane protein of the outer
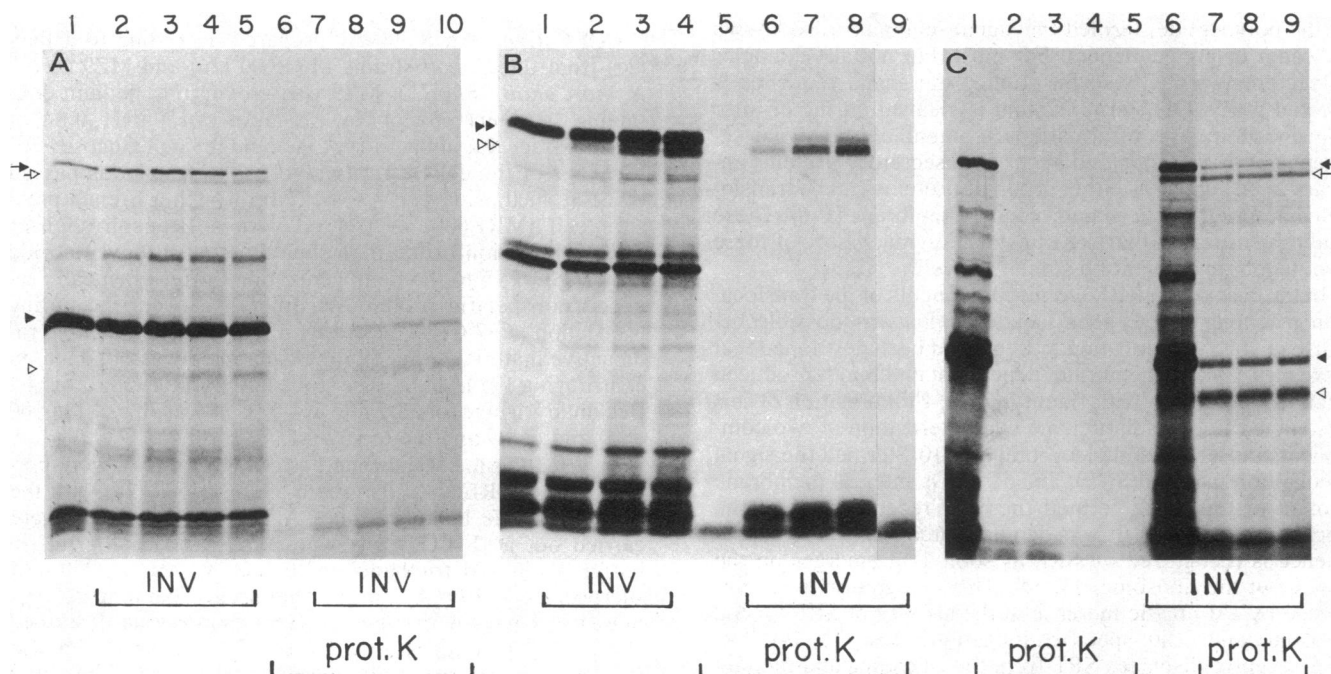

FIG. 1. *In vitro* translation and translocation into INV of alkaline phosphatase, truncated β-lactamase, and LamB protein. Plasmid pHI-1 (*A* and *C*) and plasmid pLB 7012B (*B*) were used to program a coupled transcription-translation system containing an S-135 from *E. coli* strain MZ9. Translation products were separated by polyacrylamide gel electrophoresis in NaDodSO$_4$ and visualized by fluorography. Translations were either in the absence or, where indicated, in the presence of INV. A crude INV fraction was used for experiments shown in *A* at concentrations of 0.16, 0.4, 0.8, and 1.2 A_{280} units/ml (lanes 2–5 and lanes 7–10, respectively). A purified fraction of INV was used for experiments shown in *B* at concentrations of 0.15, 0.5, and 1.0 A_{280} unit/ml (lanes 2–4 and 6–8, respectively) and in *C* at concentrations of 0.5 A_{280} unit/ml (lanes 6–9, respectively). Posttranslational proteolysis with proteinase K was carried out (where indicated) at 22°C and for 30 min at a concentration of 200 μg of proteinase K per ml. For testing a range of concentrations, proteinase K was increased to 500 μg/ml (*C*, lanes 4 and 8) or to 1000 μg/ml (*C*, lanes 5 and 9). In controls, proteinase K was added together with either Triton X-100, final concentration of 1% (wt/vol) (*B*, lane 9), or INV at a concentration of 0.5 A_{280} unit/ml (*C*, lanes 3–5). Closed symbols indicate precursors and open symbols indicate mature forms of alkaline phosphatase (arrow), truncated β-lactamase (single arrowhead), and LamB protein (double arrowheads).

membrane (Fig. 1B, lane 1, double arrowheads).

Because translocation occurs unidirectionally from the cytoplasm to the periplasmic space, in vitro reconstruction of this process requires inside-out (inverted) vesicles obtained as described (30). We tested a crude (Fig. 1A) and a sucrose-step gradient-purified preparation of INV (Fig. 1 B and C). Both were translocation-competent to about the same extent. Their presence in the cell-free system in increasing amounts yielded increasing conversion of each of these precursors to the mature forms (indicated by open symbols, Fig. 1A, lanes 2–5; Fig. 1B, lanes 2–4). However, saturation with INV was already reached when the reaction was only about 25% completed. The rate of conversion was even lower, if creatine phosphate and creatine phosphokinase were omitted (data not shown).

To verify that the membrane-dependent proteolytic processing of the three precursors reflected translocation into the lumen of the vesicles, the entire translocation mixture was subjected to posttranslational proteolysis with proteinase K. As expected, the mature forms of the three proteins (open symbols) were protected (Fig. 1A, lanes 7–10; Fig. 1B, lanes 6–8), unless the INV were disrupted by Triton X-100 (Fig. 1B, lane 9).

However, a small amount of precursor form (closed symbols) was also found to be protected. The protected precursors most likely represented translocated but unprocessed chains (i.e., not cleaved by signal peptidase) rather than incompletely digested, untranslocated chains. This conclusion is supported by the following observations: (i) in the absence of INV precursors are digested by proteinase K (200 μg/ml) (Fig. 1A, lane 6; Fig. 1B, lane 5; Fig. 1C, lane 2); (ii) when INV were added together with proteinase K to simulate a condition whereby precursor could be adsorbed to the vesicles without being translocated into them, there was no protection of precursor (Fig. 1C, lanes 3–5); (iii) higher concentrations of proteinase K (500 μg/ml, Fig. 1C, lane 8; 1000 μg/ml, Fig. 1C, lane 9) did not diminish the amount of protected precursors (nor did they affect the amount of protected mature protein).

To assess whether cleavage of precursors by our INV preparation occurred at the authentic site, we subjected the mature, in vitro-translocated, protease-resistant form of truncated β-lactamase (after immunoisolation) to partial NH$_2$-terminal sequence analysis (Fig. 2). Leucine residues were found at positions 5, 15, 24, and 26, exactly at the same positions as in the NH$_2$-terminal sequence of mature β-lactamase (31).

Previously described in vitro translocation systems led to conflicting interpretations as to whether translocation (or integration) occurs cotranslationally (16, 21, 22) or posttranslationally (20, 22, 32). Protocols to demonstrate posttranslational translocation in vitro must rule out the possibility that any observed translocation during a "posttranslational" incubation with INV might be caused by a vesicle-induced reactivation of translation. If an SRP-like component were operative also in E. coli (see Introduction), it could yield elongation-arrested chains that could be completed only after release of the arrest by added vesicles (14). Because these chains could be expected to be translocated efficiently (14) they would then be scored as posttranslationally translocated when in reality their translocation was coupled to elongation. Although attempts have been made previously to stop translation by antibiotics (32) or enzymatic digestion of the templates (20) prior to incubation with INV, in neither case has vesicle-activated translation been rigorously ruled out as a possibility for the observed posttranslational translocation.

As elongation-arrested chains would be located exclusively on ribosomes (14, 15), we decided to prepare, posttranslationally, a postribosomal supernatant. Such a supernatant, incapable of supporting translation and containing only com-
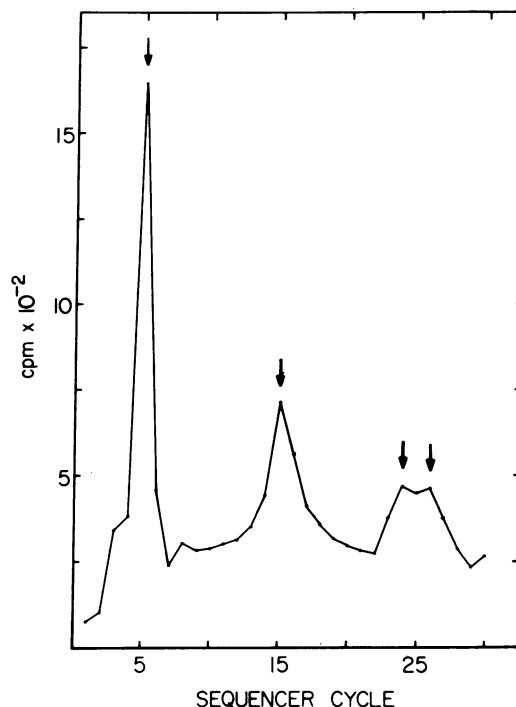

FIG. 2.   NH$_2$-terminal sequence analysis of truncated β-lactamase processed by INV. Translation was performed in a total volume of 1 ml in the presence of 2 mCi of L-[3,4,5-^{3}H]leucine by using an S-135 from the E. coli strain MRE600. After posttranslational proteolysis, the processed form of truncated β-lactamase was isolated from sedimented INV by immunoprecipitation with anti-β-lactamase antiserum (4) and subsequent polyacrylamide gel electrophoresis in NaDodSO$_4$. Preparation for automated Edman degradation was as described in ref. 4. Arrows indicate positions of leucine.

pleted chains, could be expected to contribute a more rigorously defined "substrate" in an incubation with INV to assay for posttranslational translocation. It was clear, however, that only a positive result would be definitive, as a negative result could have meant that completed chains may have rapidly lost their competence to be translocated. A loss of translocation competence within 3 min after chain completion has indeed been reported for the precursor of the major coat protein of the bacteriophage f1 (32). As can be seen from the data shown in Fig. 3, the results of our experiment were positive and therefore definitive. A posttranslational centrifugation to remove ribosomes sedimented about 50% (truncated β-lactamase) or less (alkaline phosphatase) of the precursors (lane 3), leaving the remainder in the postribosomal supernatant (lane 4). Incubation of the postribosomal supernatant with INV and a replenished energy-generating system yielded the mature forms of the two precursors (lane 6). Protection of these mature forms (and of a small amount of precursor; see above) from proteinase K digestion (lane 7) strongly suggested that they were translocated into the vesicle lumen. The efficacy of this posttranslational translocation (lanes 6 and 7) approaches that of a cotranslational translocation assay in which INV were present during translation (lanes 2 and 1). A control experiment was carried out to verify that the postribosomal supernatant was indeed translation-incompetent; translation was carried out in the presence of unlabeled methionine; a postribosomal supernatant was prepared from the translation mixture and was incubated with [^{35}S]methionine; as shown in Fig. 3, lane 8, no labeled chains were detectable under these conditions unless the sample was supplemented with ribosomes (data not shown). Thus, we conclude that translocation across the bacterial plasma membrane can occur posttranslationally.
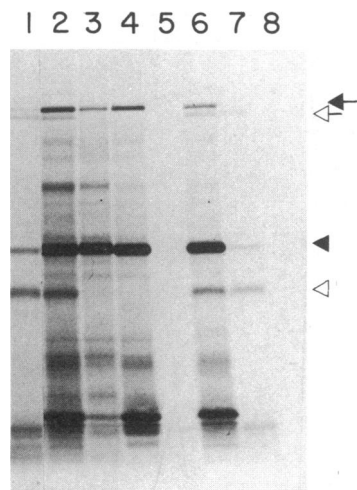
FIG. 3.   *In vitro* translocation of periplasmic proteins can occur posttranslationally. pHI-1-directed translation products are shown. Experimental details and symbols are as described in the legend to Fig. 1. Products synthesized during 30 min in the presence of purified INV (0.5 A_{280} unit/ml) are shown before (lane 2) or after (lane 1) digestion with proteinase K (200 μg/ml). Also shown are products synthesized in the absence of INV and either sedimented (lane 3) or remaining in the supernatant (lane 4) after centrifugation (at 2–4°C for 30 min at 135,000 × g in the Beckman Airfuge) through a cushion of 0.5 M sucrose in 40 mM triethanolamine acetate, pH 7.5/140 mM KOAC/11 mM Mg(OAc)$_2$/20 mM NH$_4$OAc/0.1 mM EDTA. The "supernatant" products were incubated for 30 min at 37°C with an equal volume of translation buffer containing purified INV (1.0 A_{280} unit/ml) and all of the additives listed in *Methods* (*In Vitro* Translation), except for the S-135, DNA, and [^{35}S]methionine, which were replaced by appropriate compensating buffers and a 100-fold excess of nonradioactive methionine, respectively (lane 6). Supernatant products digested with proteinase K (200 μg/ml) before (lane 5) or after (lane 7) incubation with INV are shown. A control is shown in lane 8: translation was carried out in the presence of 0.4 μM nonradioactive methionine and in the absence of INV; after the centrifugation step there was an additional incubation of the supernatant with [^{35}S]methionine.

As it took at least 30 min to prepare the postribosomal supernatant, it is clear that posttranslational translocation is not limited to a narrow time span after translation, at least not for the proteins studied in this cell-free translocation system.

DISCUSSION

The data presented here deal primarily with the development of a cell-free system from *E. coli* that is capable of reproducing protein translocation across the *E. coli* plasma membrane. This translocation system is absolutely dependent on the addition of INV. In the absence of INV, the system produces only precursors. In the presence of increasing amounts of INV, a correspondingly increasing number of precursor molecules is translocated into the vesicle lumen. Translocated chains were distinguished from untranslocated ones by probing with proteinase K. Only translocated chains were demonstrated to be resistant to proteinase K. Lysis of the protecting INV membrane by detergent abolished resistance to proteinase K.

Most of the translocated chains were found to be processed to the mature form—i.e., translocation was accompanied by removal of the signal sequence. By partial NH$_2$-terminal sequence analysis we established that cleavage occurred at the signal peptidase site for the precursor of truncated β-lactamase. The finding that cleavage of the precursors of alkaline phosphatase and the LamB protein was strictly INV-dependent and occurred almost exclusively for translocated chains (almost all processed chains were protected) suggests

that processing was caused by the signal peptidase in these two cases as well.

Surprisingly, some of the translocated chains were found to remain uncleaved. That translocation and processing of bacterial precursor proteins are not necessarily coupled events had also been demonstrated by the finding that certain signal sequence mutations affected only processing but not transport (summarized in ref. 24). The uncleaved chains probably remain anchored via their signal sequence to the INV membrane (33). The detection of translocated precursors is of importance in estimating the extent of translocation. An estimate merely based on the amount of mature chains, without probing by proteinase K, would result in an underestimation of the translocation activity.

The LamB protein of the outer membrane might be translocated across the plasma membrane and thereafter asymmetrically integrated into the outer membrane. However, it is more likely that it is asymmetrically integrated into the plasma membrane and then sorted to the outer membrane. The observed proteinase K resistance does not allow discrimination between these two possibilities, as the LamB protein might have been integrated in such a way that, under our experimental conditions, it is inaccessible to proteinase K.

Noteworthy is the observation that the addition of INV rapidly saturates the system at a level of only about 25% of the synthesized chains translocated. This indicates that some component(s) other than those contributed by INV are limiting in this system. Evidence for the existence of a soluble factor that is required for translocation in a further subfractionated system will be presented elsewhere (34).

Our finding that translocation could be improved by creatine phosphate and creatine phosphokinase points toward an energy requirement in terms of a constant replenishment of the ATP pool. It should be emphasized that increasing the ATP or creatine phosphate (or both) concentrations alone did not increase the translocation activity (data not shown).

In preparing INV care must be taken to avoid extraction of the F$_1$ portion of ATPase, as this will completely abolish the membrane's translocation activity (unpublished data).

The most important result that we have so far obtained with the present *in vitro* translocation system is the finding that translocation can occur uncoupled from translation. Translocation across the prokaryotic plasma membrane has been suggested previously to occur posttranslationally, based on studies with a cell-free translocation system (32, 35) and *in vivo* pulse–chase experiments (36–41). However, as pointed out in the *Results*, an SRP-like elongation arrest mechanism, if it were operative also in *E. coli*, might have veiled a cotranslational mode of translocation. The demonstration here of a posttranslational mode of translocation could not have been obscured by an INV-mediated release of SRP-arrested peptides and is therefore clear-cut.

It should be pointed out that our data here provide evidence only for a facultative, not an obligatory, posttranslational mode of translocation. Moreover, events preceding the actual passage across the membrane, such as recognition of the signal sequence, might be strictly coupled to translation. The latter would be consistent with genetic analyses that indicate coupling between translation and translocation (42–44).

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